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Regulation of Phytochrome B Nuclear Localization through Light-Dependent Unmasking of Nuclear-Localization Signals

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Summary

Phytochromes are red and far-red photoreceptors that regulate plant growth and development in response to environmental light cues. Phytochromes exist in two photo-interconvertible conformational states: an inactive Pr form and an active Pfr form. The alteration of phytochromes' subcellular location functions as a major regulatory mechanism of their biological activities [1–3]. Whereas phytochromes in the Pr form localize in the cytoplasm, phytochromes in the Pfr form accumulate in the nucleus, where they interact with transcription factors to regulate gene expression [1, 4]. The molecular details of the regulation of phytochrome translocation by light are poorly understood. Using *Arabidopsis* phyB as a model, we demonstrate that the C-terminal PAS-related domain (PRD) is both necessary and sufficient for phyB nuclear import and that the entire C terminus is required for nuclear-body (NB) localization. We also show that phyB's N-terminal bilin lyase domain (BLD) and PHY domain interact directly with the PRD in a light-dependent manner. In vivo localization studies indicate that BLD-PHY is sufficient to regulate phyB's nuclear accumulation. For phyB nuclear localization, our results suggest a molecular mechanism in which the nuclear-localization signal in the PRD is masked by interactions with phyB's chromophore-attachment domains and unmasked by light-dependent conformational changes.

Results and Discussion

PAS-Related Domain Is Necessary and Sufficient for phyB Nuclear Localization

Higher plant genomes encode a small family of phytochromes that respond to light quality and quantity differentially to function under various physiological conditions [1, 5, 6]. In *Arabidopsis*, phytochromes are encoded by a five-member gene family, *PHYA-PHYE* [7]. PhyB is the prominent photo-stable phytochrome involved in red-light-sensing and shade-avoidance responses [5, 6]. PhyB functions via the reversible low-fluence responses that are promoted by red (R) light and repressed by far-red (FR) light [1]. Both nuclear import and nuclear-body (NB) localization of phyB are also R/FR reversible [3, 8]. However, when phyB's N terminus (amino acids 1–600) and C terminus (amino acids 600–1172) are expressed separately as green fluorescent protein (GFP)-fusion proteins, the N-terminal fragments accumulate mostly in the cytoplasm, whereas the C-terminal fragments localize constitutively to NBs regardless of light conditions [9, 10]. These results suggest that phyB's C-terminal half contains signals for both nuclear import and NB localization; however, no nuclear-localization signal (NLS) has been identified based on primary-sequence analyses.

The C-terminal half of phytochrome consists of two recognizable subdomains, a Per-Arnt-Sim (PAS)-related domain (PRD) and a histidine-kinase-related domain (HKRD) (Figure 1A). To map the sequences involved in nuclear import and NB localization, we generated a series of C-terminal truncation fragments fused in frame with yellow fluorescent protein (YFP) and driven by the CaMV 35S constitutive promoter (Figure 1A). The localization patterns of these truncated fusion proteins were examined by a transient-expression assay in *Arabidopsis* leaf epidermal cells. As shown in Figure 1B, PB-C::YFP (amino acids 597–1172) constitutively localized to NBs, as previously reported [9, 10]. To assess the role of the PRD and HKRD in phyB localization, we generated PRD::YFP (amino acids 594–917) and HKRD::YFP (amino acids 863–1172) fusion constructs (Figure 1A). Localization assays showed that PRD::YFP localized to the nucleus; however, it failed to compartmentalize to NBs. In contrast, HKRD::YFP accumulated in cytoplasmic spots (Figure 1B and Table 1). Although the nature of the cytoplasmic spots is not clear and we cannot rule out the possibility that they are the result of misfolded HKRD::YFP fragments, the positive result for PRD::YFP localization demonstrated that PRD itself is sufficient for nuclear localization. This conclusion is consistent with a previous report that a phyB C-terminal truncation mutant (amino acids 1–990) lacking most of the HKRD still localizes predominantly to the nucleus in R light [11, 12]. This result is in contradiction with a previous one that predicted that the HKRD was required for phyB nuclear localization [13].

Because PRD::YFP did not localize to NBs, the results also suggest that the HKRD is necessary for NB localization of phyB's C terminus. To further test whether the HKRD is sufficient for NB localization, we fused an NLS from SV40 large T antigen to the C terminus of HKRD::YFP [14–16]. HKRD::YFP::NLS localized to the nucleus; however, it did not localize to NBs (Figure 1B and Table 1). This result indicates that the HKRD is not sufficient for phyB NB localization and that both the HKRD and PRD are required for localization of phyB to NBs.

To further dissect the NLS within the PRD, we generated a phyB C-terminal construct lacking the PAS-A subdomain (ΔPAS-A::YFP, amino acids 766–1172). When transiently expressed in *Arabidopsis* leaf cells, ΔPAS-A::YFP, like HKRD::YFP, localized to cytoplasmic spots. This result indicates that PAS-A (Per-Arnt-Sim) is required for phyB's nuclear import. To examine whether PAS-A is sufficient for phyB nuclear import, we fused

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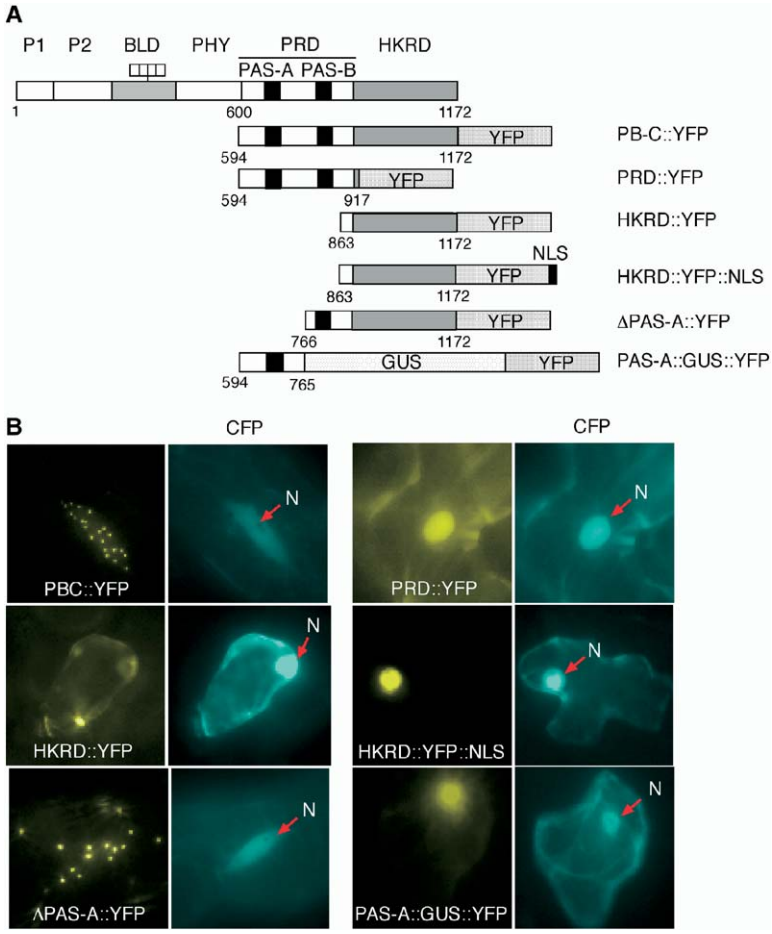

Figure 1. Mapping of NLS and NBLS in *Arabidopsis* phyB

(A) Schematic illustration of the phyB C-terminal truncation constructs.

(B) Localization patterns of the phyB fragments in *Arabidopsis* leaf transient-expression assays. All phyB fragments were fused to YFP. In the transient-expression assays, CFP was coexpressed as a marker to label the position of the nucleus. Arrows indicate the positions of nuclei (N). The localization patterns were quite robust; a representative image for each phyB fragment is presented.

PAS-A to glucuronidase (GUS) and YFP. PAS-A::GUS::YFP also localized to cytoplasmic spots (Figure 1B). There are two ways to explain the results. One explanation is that both PAS-A and PAS-B are required to form the NLS. Alternatively, it is possible that the deletion of PAS-A leads to instability and misfolding of the PRD; this instability and misfolding may prevent the NLS from being exposed. In either case, both PAS-A and PAS-B are necessary for the integrity of the NLS in the PRD.

PhyB N and C Termini Interact Directly in a Light-Regulated Manner

Because the nuclear partitioning and NB localization of full-length phyB are light regulated, we hypothesized that phyB's N-terminal domains must be able to influence C-terminal localization signals through dynamic, light-dependent structural changes. A possible mechanism for such regulation is one in which the C-terminal nuclear-localization signals are physically masked by the N-terminal domains and the exposure of these signals is light regulated, as proposed previously [2, 17]. To directly test this hypothesis, we used yeast two-hybrid assays to examine whether the N- and C-terminal halves of phyB interact with each other. The phyB N-terminal domain (amino acids 1–651) was fused to the Lex-A DNA binding domain (LBD) in a bait vector; the phyB C-terminal domain was fused to the B42-activation domain (BAD) in a prey vector (Figure 2A). The interactions were measured by liquid β-galactosidase assays. To test whether the Pr or Pfr form of the phyB N terminus has different binding affinities with the phyB

Table 1. Summary of the phyB C-terminal Truncation Fragments and Their Localization Patterns

Construct	Amino Acids	Domains	Localization
PB-C::YFP	594–1172	PRD, HKRD	NBs
PRD::YFP	594–917	PRD	nucleoplasm
HKRD::YFP	863–1172	HKRD	cytoplasm
HKRD::YFP::NLS	863–1172	HKRD::NLS	nucleoplasm
ΔPAS-A::YFP	766–1172	PAS-B, HKRD	cytoplasm
PAS-A::GUS::YFP	594–765	PAS-A	cytoplasm

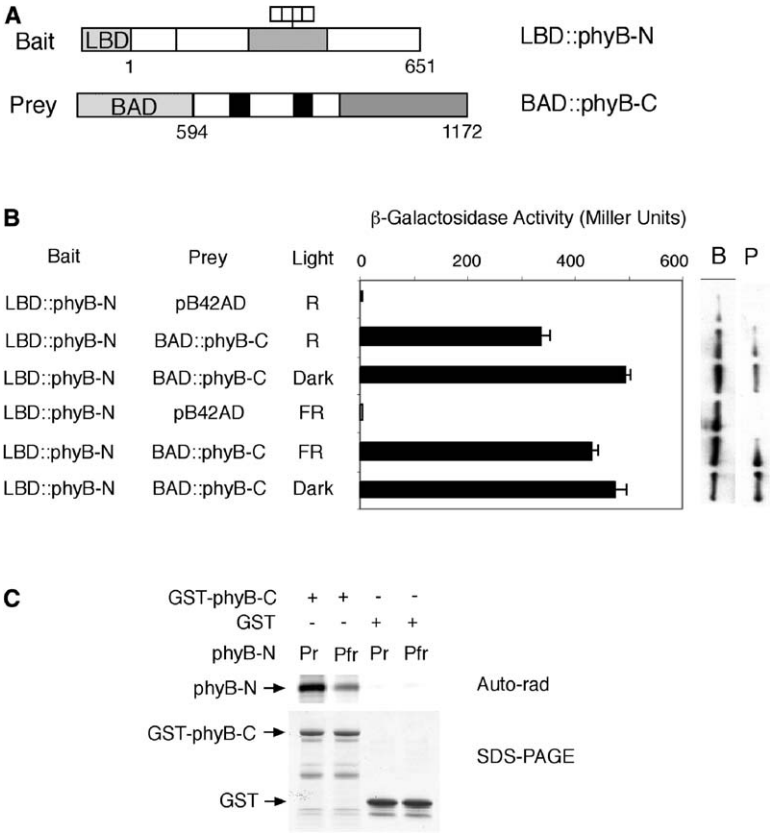

Figure 2. Light-Regulated Interactions between phyB N and C Termini

(A) Schematic illustration of phyB-N and phyB-C constructs for the yeast two-hybrid assays. The phyB-N (amino acids 1–651) was fused to the Lex-A DNA binding domain (LBD); the phyB-C (amino acids 594–1172) was fused to the B42-activation domain (BAD).

(B) Yeast two-hybrid liquid β-galactosidase activity assays between phyB-N and phyB-C in darkness and R or FR light. Yeast cells co-transformed with LBD::phyB-N and pB42AD were used as controls in the liquid β-galactosidase activity assays. Western blots show the protein levels of the bait (B) and prey (P) proteins. Error bars represent the standard deviation from 3 parallel replications.

(C) PhyB-N and phyB-C pull-down experiments. *E. coli*-expressed GST-fused phyB C-terminal fragments (amino acids 594–1172) were used to pull down in vitro-synthesized phyB N-terminal fragments (amino acids 1–651). In vitro-synthesized phyB-N fragments were first incubated with PCB to allow the covalent conjugation of PCB to phyB-N. Then the pull-down experiments were carried out in R or FR light to give rise to the Pfr or Pr form of phyB-N. GST was used as a control for the pull-down experiments. The auto-rad shows the amount of phyB-N fragments pulled down in each experiment. A corresponding SDS-PAGE gel shows the protein levels of either GST::phyB-C or GST in each experiment.

C terminus, we assembled phycocyanobilin (PCB) to phyB's N terminus prior to the liquid β-galactosidase assays [18]. We conducted liquid β-galactosidase assays in the dark, in FR light, and in R light. As shown in Figure 2B, phyB's N and C termini interacted strongly in the dark, a condition in which phyB's N terminus is in the Pr conformation. When the same assay was conducted in R light (Figure 2B, row 2), a condition that converts Pr to Pfr, the interaction was significantly and consistently weaker than that in the dark (Figure 2B, row 3). Thus, phyB's N terminus interacts with its C terminus, and light activation to the Pfr form weakens this interaction. In FR light, the interaction was slightly weaker than that in the dark (Figure 2B, rows 5 and 6). This could be due to the fact that a small fraction of phyB is in the Pfr form under FR light [19]. We also tested whether the attachment of PCB to phyB's N-terminal domain affected that domain's affinity for the C terminus. As shown in Figure S1A (in the Supplemental Data available with this article online), PCB attachment alone has no significant effect on the interaction.

To further confirm the R/FR-dependent interaction between phyB N and C termini, we performed in vitro pull-down assays with an *E. coli*-expressed GST::phyB C-terminus (amino acids 594–1172) fusion protein as the bait and an in vitro-translated, ^{35}S-methionione-labeled, PCB-conjugated phyB N terminus (amino acids 1–651) as the prey. Again, the binding assays were carried out in either R or FR light to assess the different binding properties of the Pfr and Pr forms of phyB's N terminus. Consistent with the yeast two-hybrid assays, the in vitro pull-down assays showed that phyB's C terminus interacted with the Pr form of phyB's N terminus twice as strongly as with its Pfr form (Figure 2C).

The Interaction between N and C Termini of phyB Involves Mainly the BLD-PHY Domains and PRD

To investigate the role of the PRD in the intramolecular interaction between phyB's N and C termini, we tested the PRD and HKRD separately for the interaction with the phyB N terminus (Figure 3A). Because the Pr form of phyB's N terminus interacts with the C terminus with a higher affinity than the Pfr form does, the yeast two-hybrid interaction assays were performed in the dark, a condition in which phyB was in the Pr form. As shown in Figure 3B, PRD alone interacted with phyB's N terminus quite strongly (Figure 3B, rows 4 and 6), whereas the interaction between the HKRD and phyB's N terminus was only about 10% as strong as that between phyB's C and N termini (Figure 3B, rows 4 and 8). Moreover, PRD itself also interacted with the Pr form with a higher affinity than with the Pfr form (Figures S1B and S1C). These results indicate that phyB's N terminus interacts with the C terminus largely through the PRD.

To ascertain the precise N-terminal region that is responsible for the light-regulated interaction between phyB N- and C-terminal domains, we performed in vitro pull-down assays with *E. coli*-expressed GST::phyB C terminus as the bait and in vitro-synthesized, ^{35}S-methionine-labeled N-terminal fragments as the prey. Deletion of the P1 domain or of both the P1 and P2 domains in phyB-NΔ75 (amino acids 76–651) and phyB-

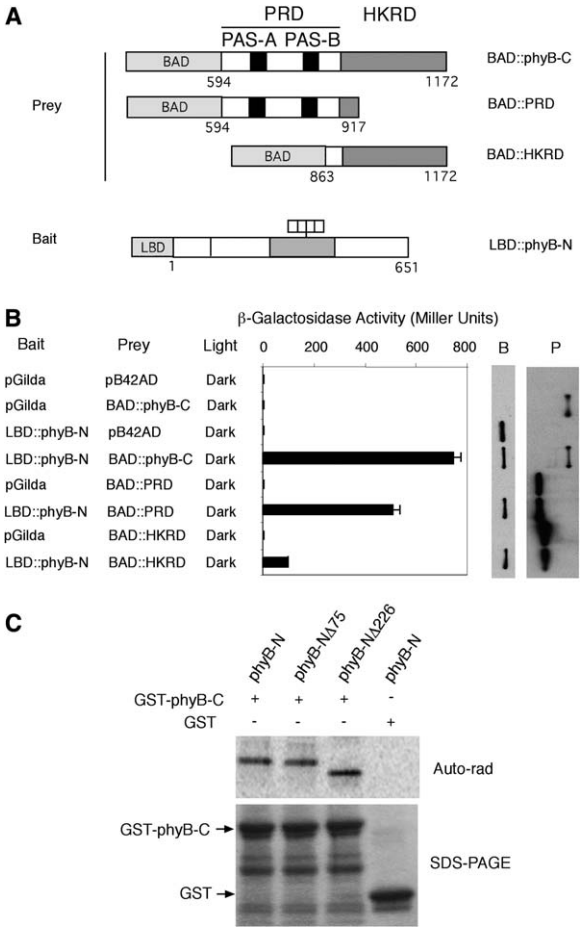

Figure 3. phyB N Terminus Interacts Mainly with the PRD

(A) Schematic illustration of phyB-N and phyB C-terminal truncation constructs for yeast two-hybrid assays.

(B) Yeast two-hybrid liquid β-galactosidase activity assays between phyB-N and phyB C-terminal fragments. Error bars represent the standard deviation from 3 parallel replications.

(C) The interactions between PCB-conjugated phyB N-terminal fragments and the phyB C terminus were evaluated with in vitro pull-down assays. *E. coli*-expressed GST-fused phyB C-terminal fragments were used to pull down in vitro-synthesized, ^{35}S-methionine-labeled phyB N-terminal fragments in the dark.

NΔ226 (amino acids 226–651) did not affect their affinities to GST::phyB-C (Figure 3C, lanes 1 and 2). Neither phyB-NΔ75 nor phyB-NΔ226 could be pulled down by GST alone (data not shown). These results suggest that the bilin lyase domain (BLD) and PHY (the phytochrome apoprotein) subdomains of phyB's N terminus are sufficient for the interaction with phyB's C terminus. We used yeast two-hybrid assays to verify the results. As shown in Figure S2, LBD::phyB-NΔ75 interacted with BAD::phyB. However, LBD::phyB-NΔ226 did not interact with BAD::phyB in the yeast two-hybrid assays. This might be explained by the misfolding of LBD::phyB-NΔ226 fusion proteins in the yeast system.

BLD-PHY Subdomains Are Sufficient for Regulating the Nuclear Import of phyB

If the interaction between phyB N and C termini is the underlying mechanism that governs the light-regulated exposure of the NLS in the PRD, the region (amino acids 227–651) containing the BLD and PHY subdomains should be sufficient to regulate nuclear localization in a light-dependent manner. To test this in vivo, we made three additional constructs: the full-length phyB fused to CFP (PBC), phyB P1 deletion fused to YFP (PBΔ75::YFP, amino acids 76–1172), and phyB P1 and P2 deletion fused to YFP (PBΔ226::YFP, amino acids 226–1172) (Figure 4A). Each construct was expressed under the control of the CaMV 35S promoter and was transformed into a *phyB* null allele, *phyB-9*. Homozygous transgenic lines were selected and used for further characterization. In the dark, the majority of PBC proteins were localized in the cytoplasm. Under 8 μmole^{-2}s^{-1} of R light, PBC proteins accumulated in the nucleus, where they compartmentalized predominantly into NBs (Figure 4B) [11, 18]. PBΔ75::YFP showed localization patterns similar to those of PBC in the dark. In contrast, in R light, PBΔ75::YFP proteins accumulated in the nucleus and yet failed to localize to NBs (Figure 4B). This result suggests that the P1 domain is critical for NB localization of full-length phyB but is not required for nuclear accumulation of phyB. When both the P1 and P2 domains were deleted, PBΔ226::YFP proteins also showed predominant cytoplasmic localization in the dark and nucleoplasmic localization in R light. The localization patterns of PBΔ226::YFP demonstrate that the BLD and P4 domains are sufficient to regulate nuclear import of phyB in a light-dependent manner. Consistent with a previous report [20], neither PBΔ75::YFP nor PBΔ226::YFP is functional despite their nuclear localization in R light (Figures S3A and S3B).

Together, these data provide new evidence to support a NLS-unmasking model for phyB's light-regulated nuclear localization (Figure 4C). In the Pr form, the NLS in the PRD is masked through direct interactions with the BLD and PHY subdomains of the N terminus. In the Pfr form, structural rearrangements in BLD and PHY lead to weaker interactions with the PRD, allowing the exposure of the NLS. Consistent with the model, previous HNB-Br modification experiments suggested that two Trp residues in the PAS-B domain are preferentially exposed in the Pfr form [17]. It is also possible that the interaction between phyB's N and C termini occurs through an intermolecular interaction between monomers in the homodimer. The conformational changes of phytochrome in the Pfr and Pr states might also be important to regulate the topography of phyB's N terminus and its binding affinities to other signaling molecules, such as PIF3 [21]. Through this mechanism, the regulation of both phytochrome's localization and signaling is directly linked to environmental light cues.

Supplemental Data

Supplemental data including detailed Supplemental Experimental Procedures and three figures are available at http://www.current-biology.com/cgi/content/full/15/7/637/DC1/.

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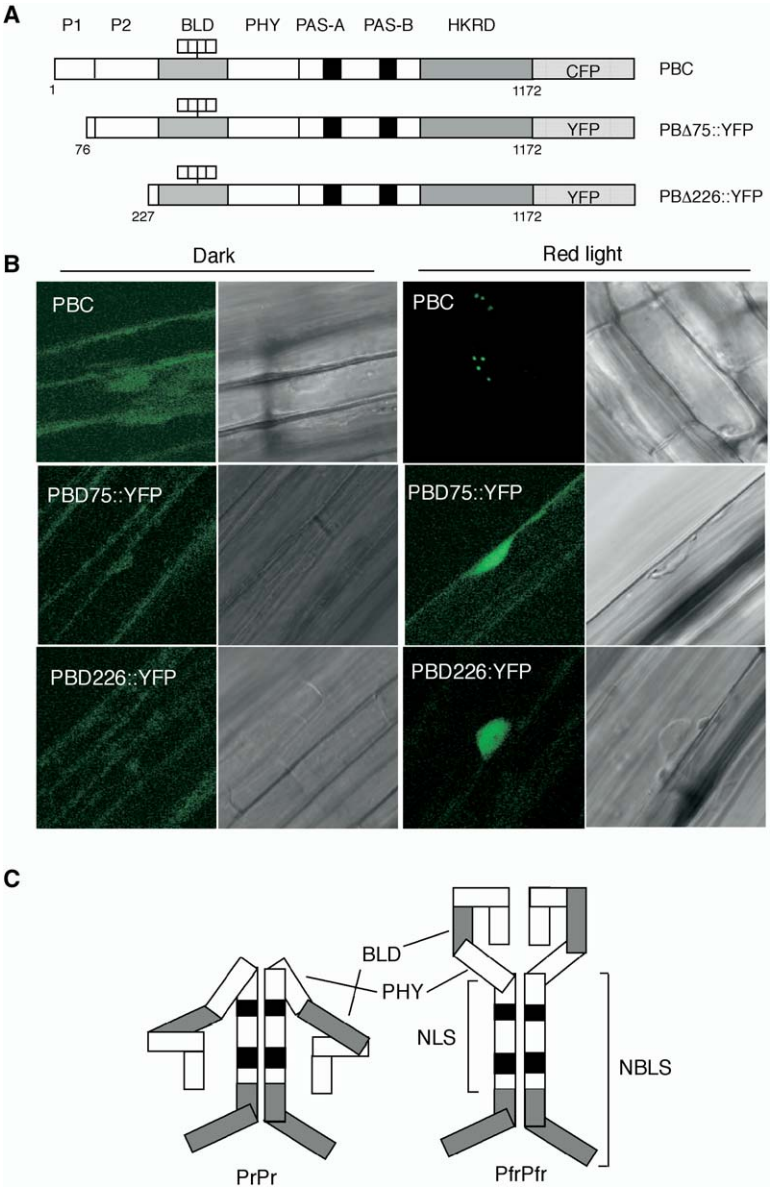

Figure 4. BLD and PHY Are Sufficient for Light-Dependent Regulation of phyB Nuclear Localization

(A) Schematic illustration of phyB::CFP (PBC), PBΔ75::YFP, and PBΔ226::YFP.

(B) In vivo localization patterns of PBC, PBΔ75::YFP, and PBΔ226::YFP in hypocotyl cells of transgenic lines either in the dark (two lefthand columns) or in R light (two righthand columns). DIC images are shown to indicate the position of the nucleus.

(C) Schematic model depicting intramolecular interactions in phyB for light-regulated nuclear import. It should be noted that intermolecular interactions between monomers in the homodimers are also possible.

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